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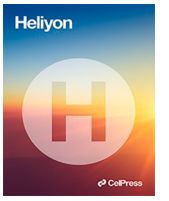

Research article

Peimine promotes microglial polarization to the M2 phenotype to attenuate drug-resistant epilepsy through suppressing the TLR4/NF-κB/HIF-1α signaling pathway in a rat model and in BV-2 microglia

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ABSTRACT

Epilepsy is a chronic neurological disorder. Drug-resistant epilepsy (DRE) accounts for about one-third of epilepsy patients worldwide. Peimine, a main active component of Fritillaria, has been reported to show anti-inflammatory effects. However, its potential therapeutic role in DRE is not yet fully understood. In this work, a DRE rat model was established by injecting 1 μg kainic acid (KA), followed by a 250 mg/kg administration of valproic acid (VPA) from day 4–31. Rats were treated with different doses of peimine (2.5 mg/kg, 5 mg/kg and 10 mg/kg) daily from day 32–62. *In vitro*, BV-2 microglia were exposed to different doses of peimine (7.5 μg/ml, 15 μg/ml, and 30 μg/ml) in presence of LPS. The aim of this study was to investigate the potential therapeutic effects of peimine on DRE. The results showed that peimine efficiently suppressed the KA-induced epileptic behaviors of rats in a dose-dependent manner, as recorded by electroencephalography. Furthermore, peimine ameliorated hippocampal neuron injury in DRE rats, and promoted an M1-to-M2 microglial phenotype shift in a dose-dependent manner. Mechanistically, peimine inhibited the TLR4/NF-κB/HIF-1α signaling pathway both *in vivo* and *in vitro*. Additionally, peimine suppressed the apoptosis of primary neurons induced by LPS-treated microglia. In conclusion, peimine augments the microglial polarization towards an M2 phenotype by inhibiting the TLR4/NF-κB/HIF-1α signaling pathway, thereby attenuating DRE.

1. Introduction

Epilepsy, a multifaceted neurological disease, is characterized by recurrent spontaneous seizures [1]. Although antiepileptic drugs

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(AEDs) are currently effective, nearly 30 % of patients with epilepsy are refractory to drug treatment and are accompanied by progressive cognitive dysfunction, which may require neurosurgical resection of epileptic lesions to improve epilepsy recurrence [2,3]. Notably, drug-resistant epilepsy (DRE) has become a thorny problem in clinical epilepsy, especially in temporal lobe epilepsy (TLE) [4]. Over the past decade, studies in human epilepsy patients have suggested that neuroinflammatory processes are involved in the process of epilepsy and play a vital role in neuronal hyperexcitability on the basis of seizures [5]. Therefore, the development of new therapeutic targets targeting neuroinflammatory signaling pathways may provide the basis for its clinical strategies.

Peimine is the main compound extracted from Fritillaria. Fritillaria, a traditional Chinese medicine, has been used as a cough and anti-asthma agent because of its high efficacy and few side effects [6]. In cancers, peimine exhibits an anti-tumor role, along with an inhibition in the growth and motility of prostate and breast cancer cells [7–9]. In addition, peimine has anti-inflammatory effects on multiple diseases. For instance, peimine suppressed inflammation caused by IL-1β in mouse chondrocytes via repressing the MAPK pathway, thus alleviating osteoarthritis [10]. Also, peimine inhibited the secretion of proinflammatory cytokines by inactivating the NF-κB and MAPK pathways in HMC-1 cells [11]. Peimine also relieved rheumatoid arthritis via suppressing collagen-induced arthritis and TNFα-activated MAPK pathways [12]. In addition, peimine downregulated inflammatory cytokine levels and upregulated anti-inflammatory cytokine levels in LPS-stimulated RAW 264.7 macrophages [6]. Microglia are common macrophages in the central nervous system, mediating the onset and progression of neuroinflammation [13]. Microglial modifications involved pro-inflammatory (M1) and anti-inflammatory (M2) markers [14]. Strikingly, the microglial phenotype shift from M2 to M1 is vital in the process of neurological diseases. Accumulating evidence indicates that inducing microglial M2 phenotype polarization is a valuable neuroprotective method in epileptic pathology [15–17]. Therefore, we explored whether peimine promotes M2 polarization of microglia to alleviate neuroinflammation.

TLR4 is expressed in microglia and induces the activation of microglia [17]. TLR4 leads to the activation of NF-κB via MyD88/TRIF signaling pathway, thus inducing inflammatory response [18]. Of note, peimine restrained inflammation in mice with acute lung injury by inhibiting TLR4/MAPK/NF-κB pathway [19]. In addition, the TLR4/NF-κB/HIF-1α signaling was demonstrated to be involved in inflammatory processes [20]. Plausibly, we explored whether peimine regulates M2 polarization of microglia through the TLR4/NF-κB/HIF-1α signaling pathway.

In our study, we utilized a rat model induced by kainic acid (KA) and valproic acid (VPA), as well as a cell model induced by LPS, to examine the potential impact of peimine on the pathogenesis of DRE. The specific objectives of this study were to investigate: (1) the effects of peimine on M1/M2 polarization and neuroinflammation in microglia; (2) its effects on neuronal apoptosis; and (3) its effects on the TLR4/NF-κB/HIF-1α signaling pathway.

2. Materials and methods

2.1. Animals and model

Animal procedures were in line with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The study was approved by Ethics Committee of the First Affiliated Hospital of Henan University of Chinese Medicine (IACUC-202301003). Male Sprague Dawley (SD, 6 weeks of age) rats with a body weight of 180–200 g were adaptively housed for 1 week. TLE model was induced by KA (MACKLIN, Shanghai, China) according to a previous method. Briefly, rats were anesthetized with isoflurane and placed in a stereotaxic apparatus [21]. The dose of 1 μg kA (1 μg kA dissolved in 1 μl saline) was injected into the right ventricle (0.2 mm posterior to the anterior fontanelle, 1.5 mm from the midline and 4.2 mm under the dura) at a rate of 0.1 μl/min. Sham-operated rats were injected with the same volume of saline [22]. Racine scale was used to evaluate epileptic seizures 3 days after KA injection. Status epilepticus (SE) is defined as a seizure of grade IV or above, and seizures last for more than 30 min. When the duration of SE exceeded 90 min, the seizures stopped after intraperitoneal injection of 10 mg/kg diazepam.

For pharmacological evaluation, 3 days after KA injection, rats were given 250 mg/kg VPA orally once a day for 4 weeks. After the last administration of VPA (day 31), seizure latency and intensity scores were recorded. Seizure intensity was scored according to the Racine scale [23]. Finally, rats with seizures were screened for inducing DRE. The dose of peimine was referred to a previous literature [24]. DRE rats were orally gavage with different doses of peimine (2.5 mg/kg, 5 mg/kg and 10 mg/kg) once a day for 30 days. Sham rats and DRE rats were respectively given equal volume solvent. One day after the last administration of peimine (day 62), the electroencephalography (EEG) of rats was recorded. Then rats were sacrificed, and hippocampal tissues were collected.

2.2. Coculture

Mouse microglial BV-2 cell line was purchased from iCell (Shanghai, China). Cells were maintained in DMEM (Servicebio, Wuhan, China) containing 10 % FBS (Tianhang Biotechnology, Zhejiang, China) in a 37 °C, 5 % CO_2 incubator. The dose of peimine was referred to an earlier literature [6]. Different doses of peimine (7.5 μg/ml, 15 μg/ml and 30 μg/ml) were added 1 h prior to 1 μg/ml LPS treatment. Then BV-2 cells were cocultured with peimine and LPS for 24 h according to a previous study [25]. Besides, after 24 h of culture with fresh medium, the conditioned medium of BV-2 was harvested.

2.3. Fluoro-Jade B (FJB) staining

Embedded hippocampal tissues were cut into 5 μm thick sections. Following the instructions of the FJB staining kit (Merck Millipore, USA), sections were treated with 0.06 % potassium permanganate solution for 10 min and stained with FJB solution at room

temperature for 20 min. The staining was captured by a microscope (Olympus, Japan).

2.4. Immunohistochemistry (IHC) staining

Sections were treated with 3 % H_2O_2 to eliminate the endogenous peroxidase activity and blocked with 1 % BSA for 15 min. Then sections were incubated with primary antibodies TLR4 (1:50, AF7017, Affinity, Changzhou, China) or NeuN (1:50, 26975-1-AP, Proteintech, Wuhan, China) at 4 °C overnight, followed by HRP-labeled goat anti-rabbit IgG secondary antibody (1:100, SE134, Solarbio, Beijing, China) at 37 °C for 60 min. Next, sections were stained with DAB (Sangon Biotech, Shanghai, China) and counterstained with hematoxylin (Solarbio). Images was captured by a microscope.

2.5. TUNEL staining

To detect the apoptosis in rat hippocampus, sections were incubated with 0.1 % Triton X-100 (50 μl, Beyotime, Shanghai, China) for 8 min. TUNEL reaction solution (50 μl) was added to the sections and incubated at 37 °C for 60 min in the dark. In addition, to detect the apoptosis in BV-2 cells, cells were permeated in 0.1 % Triton X-100 (200 μl, Beyotime) and incubated with TUNEL reaction solution (50 μl) at 37 °C for 60 min in the dark. Then sections were stained with DAPI (Aladdin, Shanghai, China) for 5 min. Images were acquired by a microscope.

2.6. Timm staining

Paraffin sections of embedded neural tissues were stained with Timm according to the instructions of the Timm staining kit (GENMED, Shanghai, China). Briefly, sections were treated 200 μl GENMED staining solution and incubated at room temperature for 80 min until black appearance. Then the GENMED staining solution was removed. Then sections were incubated with 50 ml GENMED cleaning solution at room temperature for 5 min. After removing the cleaning solution, the staining results were photographed by a microscope.

2.7. Flow cytometry

Tissues were filtered with a 200-μm filter to obtain total cells. Cells were washed with PBS, and single cell suspension was collected. Approximately (1×10^6) cells were added to each tube, and the volume was 100 μl. CD11b antibody (0.5 μg, FITC-65229, Proteintech) and CD16 antibody (0.5 μg, b101668-CF488A, Biorbyt, UK) were added for CD11b + $CD16^+$ detection. 0.5 μg CD11b antibody and 5 μl CD86 antibody (F3108602, Liankebio, Hangzhou, China) were added for CD11b + $CD86^+$ detection. 0.5 μg CD11b antibody and CD163 antibody (1:60, ab182422, Abcam, Shanghai, China) were added for CD11b + CD163+ detection. 0.5 μg CD11b antibody and CD206 antibody (1:1000, 18704-1-AP, Proteintech) were added for CD11b + CD206+ detection. Besides, for $CD16^+$ or CD206+ detection, cells were incubated with 0.5 μg CD16 antibody or 5 μl CD206 antibody. All antibodies were incubated in the dark at 4 °C for 30 min. Cells were washed with PBS and suspended with 500 μl buffer. Flow cytometry was conducted with the corresponding channel with a NovoCyte flow cytometer (Agilent, USA).

2.8. CCK-8 assay

The primary hippocampal neurons were seeded into the 96-well plates at 1×10^4 cells/well, and treated with the conditioned medium of BV-2 cells (1:1 vol ratio). CCK-8 reagent (KeyGEN Biotech, Nanjing, China) was added to the plates and incubated for 2 h. OD values at 450 nm were examined using a microplate reader (Biotek, USA).

2.9. Quantitative real time PCR (qRT-PCR)

RNA was extracted with TRIpure (Biotek, Beijing, China) and reverse transcribed to cDNA with reverse transcriptase (BeyoRT II M-MLV, Beyotime, Shanghai, China). qRT-PCR was conducted by a SYBR green kit (Solarbio, Beijing, China) with a qRT-PCR reaction system (ExicyclerTM96, BIONEER, Korea). The $2^{-\Delta\Delta Ct}$ method was used for data quantification. The primer sequences are listed in

Table 1
qRT-PCR primer sequences.

name	sequence 5′-3′	tm
Arg1 F	GCTGGCTGCTGTGGTAG	51.9
Arg1 R	ACGCATAGGTCAGGGTG	50.9
CD86 F	GGGATAACCAGGCTCTA	46.9
CD86 R	TGTTGTCGCCATACTCA	47.1
CD206 F	ATCTGGAGGCTGATTACG	49.9
CD206 R	GGTGTAGGCTCGGGTAG	50.1
iNOS F	TCCCGAAACGCTACACTT	53.6
iNOS R	CGGCTGGACTTCTCACTCT	54.6

Table 1.

2.10. Western blot

Hippocampal tissues or cells were lysed in RIPA buffer (Beyotime). BCA protein assay kit (Beyotime) was to detect protein concentration. Samples (30 μg) were separated on 10 % SDS-PAGE and transferred to PVDF membranes (Thermo Fisher Scientific, USA). The membranes were incubated with primary antibodies at 4 °C overnight. The following day, the membranes were exposed to the secondary antibodies (HRP-labeled goat anti-rabbit IgG, 1: 10000, Proteintech or HRP-labeled goat anti-mouse IgG, 1: 10000, Proteintech) at 37 °C for 40 min. Next, the membranes were treated with ECL (7 Sea Biotech, Shanghai, China), and data were quantified using Gel-Pro-Analyzer. The primary antibodies are listed in Table 2.

2.11. Quantification of cytokine levels

IL-6 and IL-10 levels were estimated based on the instructions of respective mouse ELISA kits (Liankebio). Briefly, 100 μl standard substances or 100 μl samples were added into each well. Afterwards, 50 μl detection antibody was added into each well and incubated at room temperature for 1.5 h. After washing, 100 μl horseradish peroxidase labeled streptavidin was added to each well. After incubation for 30–45 min and washing, each well was added with 100 μl chromogenic substrate TMB. Following incubation in the dark for 5–30 min, 100 μl termination solution was added into each well to terminate the reaction. Later, the OD values at 450 nm detection wavelength and 570 nm reference wavelength were determined. The IL-6 and IL-10 concentrations were calculated according to the standard curve which was established by the OD values and concentrations of standard substances.

2.12. Statistical analysis

Data (mean ± SD) were analyzed using GraphPad software (GraphPad, USA). $p < 0.05$ was considered statistically significant. Statistical differences from multiple groups were conducted with one-way analysis of variance, followed by Tukey test.

3. Results

3.1. Peimine alleviated epileptic behaviors of DRE rats

As shown in Fig. 1A, the KA-induced DRE rat model was established. After VPA administration according to KA-induced seizure behaviors, we administered low-dose, medium-dose and high-dose of peimine to the DRE rats to explore its role during DRE. Spontaneous recurrent seizures (SRS) are considered to be the spectrum of focal seizures that can be secondarily generalize [26]. In this work, video-EEG recording was used to analyze SRS and epileptiform spike rate one day after peimine administration. Noteworthily, SRS was not observed in control rats. Whereas, strongly SRS frequency occurred in rats receiving KA. Compared with the DRE rats, the abnormal SRS of DRE rats were greatly alleviated by peimine in a dose-dependent manner (Fig. 1B). Also, there were almost no spikes in control rats, while the count and amplitude of the spike were markedly increased in DRE rats and decreased by peimine simulation (Fig. 1C). In addition, representative EEG was presented in Fig. 1D. Totally, these observations showed that peimine alleviated epileptic behavior of DRE rats.

3.2. Peimine inhibited hippocampal injury of DRE rats

To investigate the effects of peimine on neuron degeneration in CA3 region of hippocampus, FJB (a key marker for neuron

Table 2
Primary antibody information.

name	dilution rate	cat. number	manufacturers
iNOS antibody	1: 500	AF0199	Affninty
TNF-α antibody	1: 1000	A0277	ABclonal
IL-6 antibody	1: 1000	A0286	ABclonal
Arg1 antibody	1: 1000	DF6657	Affninty
IL-4 antibody	1: 1000	A14660	ABclonal
IL-10 antibody	1: 500	A2171	ABclonal
TLR4 antibody	1: 500	AF7017	Affninty
MyD88 antibody	1: 500	AF5195	Affninty
p-IκBα antibody	1: 500	AF2002	Affninty
IκBα antibody	1: 500	AF5002	Affninty
p-NF-κB p65 antibody	1: 500	AF2006	Affninty
NF-κB p65 antibody	1: 500	AF5006	Affninty
HIF-1α antibody	1: 2000	BF8002	Affninty
Bcl-2 antibody	1: 500	A0208	ABclonal
Bax antibody	1: 500	A0207	ABclonal

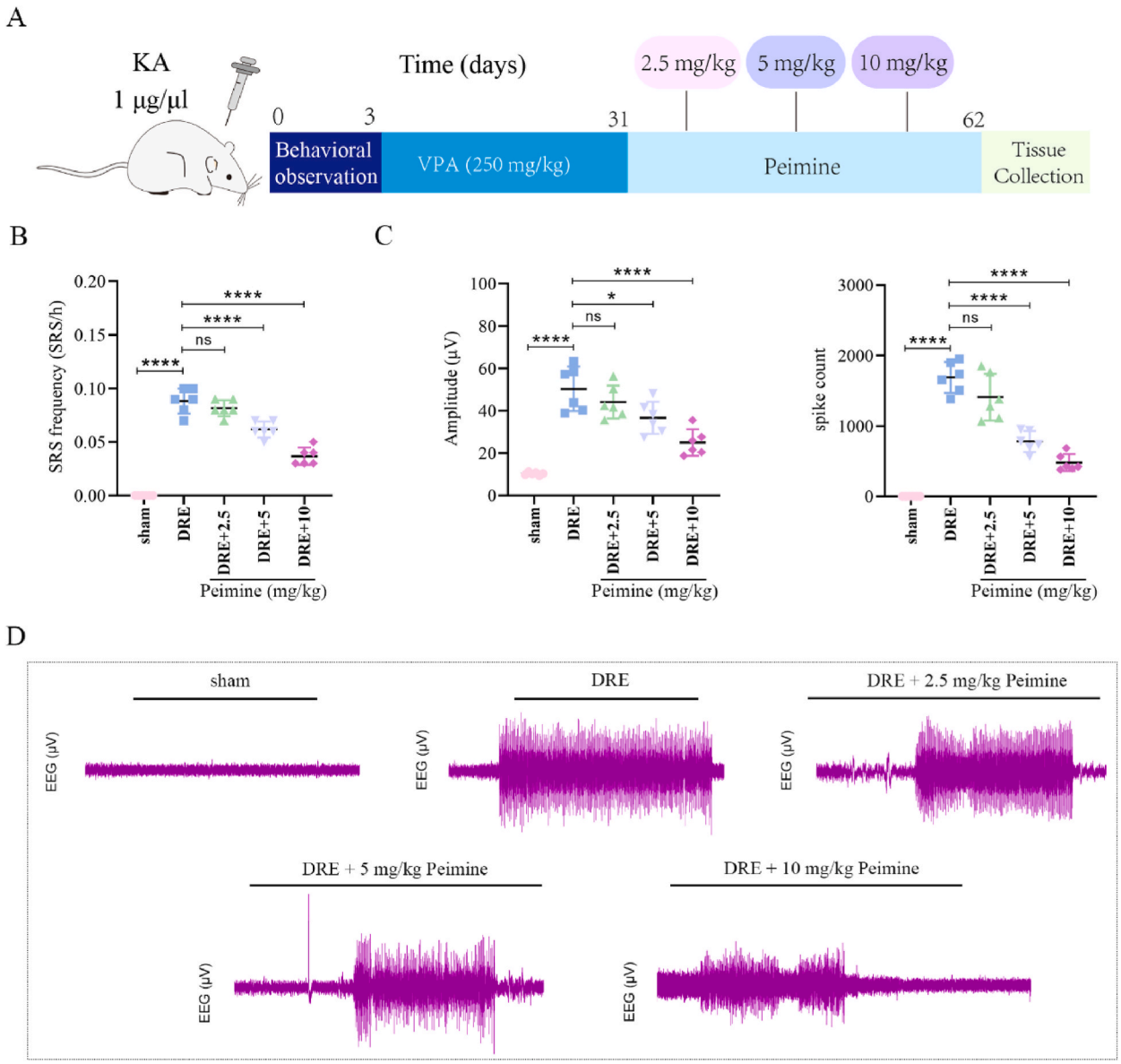

Fig. 1. Peimine alleviated epileptic behaviors of DRE rats. (A) Schematic diagram of the experimental design. (B) SRS frequency among groups. (C) Graphs quantifying seizure parameters (amplitude and spike count). (D) Representative EEG traces. *, $p < 0.05$; ****, $p < 0.0001$.

degeneration) staining was performed. As shown in Fig. 2A, controls showed no FJB-positive cells. But many degenerating cells were observed in DRE rats. Compared to the DRE rats, the degree of neuronal injuries was significantly decreased after peimine administration. Moreover, IHC staining of NeuN was employed to estimate neuron loss in the CA3 region of rat hippocampus. Abundant NeuN-positive cells were found in the control rats. Whereas, neuron loss occurred in DRE rats, which was alleviated by peimine treatment (Fig. 2B). TUNEL staining results suggested that there was little neuronal apoptosis in the controls, while DRE rats had more TUNEL-positive cells. Whereas, peimine significantly inhibited DRE-induced neuronal apoptosis (Fig. 2C). Timm staining results showed that enhanced mossy fiber sprouting was observed in DRE rats, but not in controls (Fig. 2D). Compared to the DRE group, less mossy fiber sprouting was found in peimine-treated DRE rats (Fig. 2D). To summarize, these results provided evidence that peimine treatment showed neuroprotective effects in a DRE rat model.

3.3. *Peimine promoted an M1-to-M2 microglial phenotype shift in hippocampal microglia of DRE rats*

To validate whether peimine affects microglia polarization, we detected the expression of M1 and M2 phenotype microglia markers in the hippocampus of rats. qRT-PCR results indicated that the mRNA levels of M1 polarization markers (iNOS and CD86) were

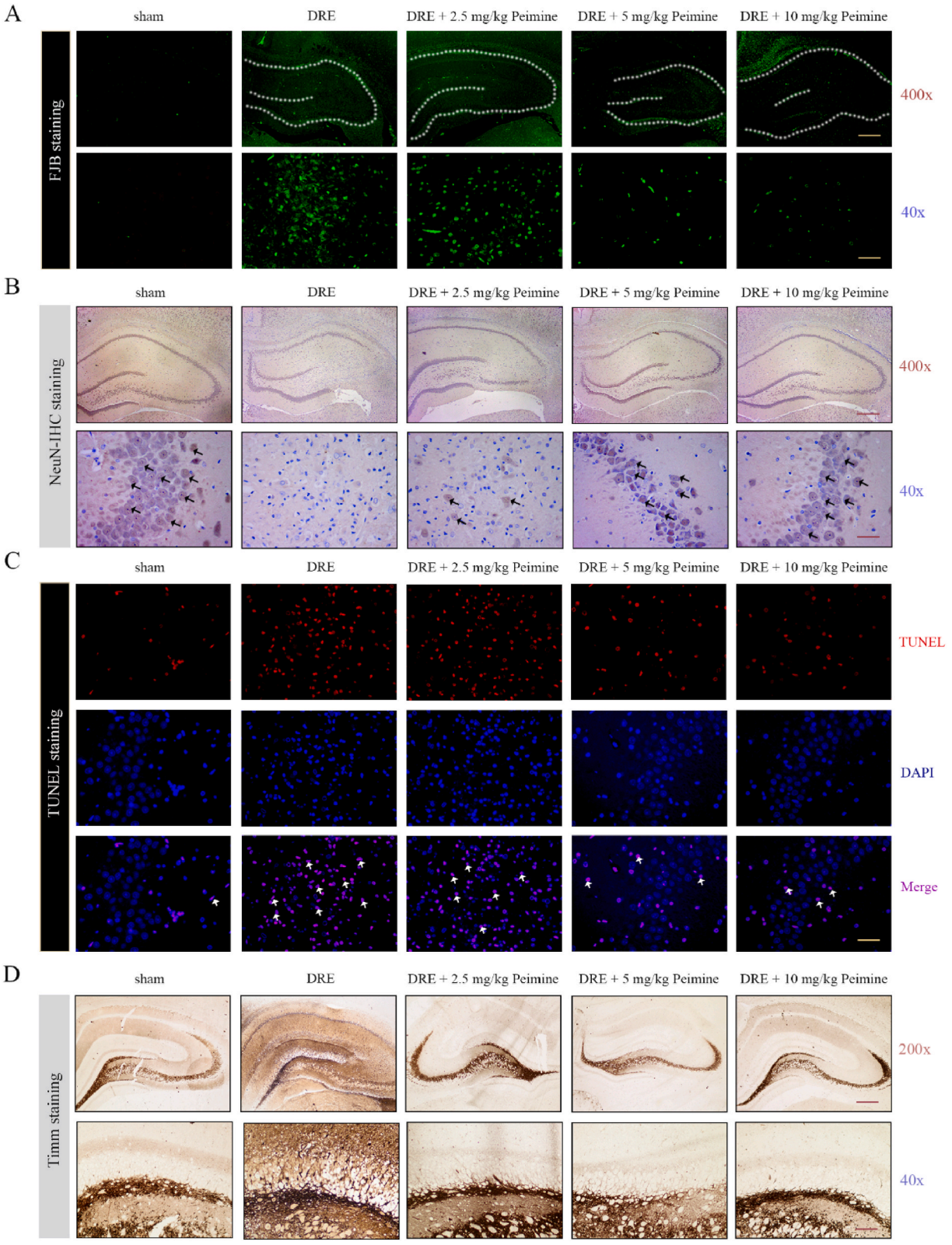

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Fig. 2. Peimine inhibited hippocampal injury of DRE rats. (A) FJB staining was used to detect neuronal damage in the CA3 area of rat hippocampus (400 ×, scale bar = 50 μm; 40 ×, scale bar = 500 μm). (B) NeuN IHC staining was employed to determine neuron loss in the CA3 area of rat hippocampus (400 ×, scale bar = 50 μm; 40 ×, scale bar = 500 μm, the arrows represent NeuN-positive cells). (C) TUNEL staining was applied to measure apoptosis in rat hippocampus (scale bar = 50 μm; the arrows represented TUNEL-positive cells). (D) Timm staining was used to examine mossy fiber sprouting in rat hippocampus (200 ×, scale bar = 100 μm; 40 ×, scale bar = 500 μm).

remarkably increased in DRE rats, while peimine stimulation suppressed their expression in a dose-dependent manner (Fig. 3A). Additionally, the mRNA levels of M2 polarization markers (Arg1 and CD206) were significantly decreased in DRE rats, while increased by peimine treatment (Fig. 3A). As shown in Fig. 3B–C, the expression of M1 microglial markers (CD16/CD86) was largely elevated in DRE rats, while DRE rats given peimine showed an opposite expression trend. Furthermore, DRE induced a considerable decrease in the expression of M2 microglial markers (CD163/CD206), which was reversed by peimine administration in a dose-dependent manner (Fig. 3B–C). These data suggested that peimine promoted an M1-to-M2 microglial phenotype shift in hippocampal microglia of DRE rats.

To further identify the above observations, we measured levels of the M1 pro-inflammatory cytokines (iNOS, TNF-α and IL-6) and M2 anti-inflammatory cytokines (Arg1, IL-4 and IL-10). As illustrated by Western blot, peimine administration notably decreased the levels of M1 markers and increased the levels of M2 markers in a dose-dependent manner, thus reversing the effects of DRE on these indexes (Fig. 3D–E). In total, these data confirmed that peimine promoted microglial M2 polarization, therefore showing anti-inflammatory effects.

3.4. Peimine inactivated the TLR4/NF-κB/HIF-1α signaling pathway in hippocampus of DRE rats

To identify whether peimine regulates microglial polarization via the TLR4/NF-κB/HIF-1α signaling pathway, related protein levels were detected by Western blot. The analysis manifested that the DRE rats showed higher protein levels of TLR4, MyD88, nuclear NF-κB p65 and HIF-1α, and phosphorylation of IκB-α and NF-κB p65 than controls. However, DRE-induced the activation of the TLR4/NF-κB/HIF-1α pathway was inhibited following dose-dependent peimine treatment (Fig. 4A). Besides, IHC staining presented that TLR4 expression was notably increased in DRE rats, while decreased in peimine-treated DRE rats (Fig. 4B), which was consistent with the above analysis. Together, peimine promoted M2 polarization in hippocampal microglia of DRE rats by inactivating the TLR4/NF-κB/HIF-1α signaling pathway.

3.5. Peimine promoted microglial M2 polarization by inhibiting the TLR4/NF-κB/HIF-1α signaling pathway in LPS-treated microglia

To further evaluate the therapeutic effects of peimine on DRE *in vitro*, BV-2 microglia were stimulated with different doses of peimine and LPS. As illustrated, LPS significantly increased the expression of CD16, but had no effects on CD206. Furthermore, LPS increased M1 marker IL-6 levels, but had no effects on M2 marker IL-10 levels. Importantly, peimine treatment dose-dependently decreased the levels of CD16 and IL-6, and increased the levels of CD206 and IL-10 in LPS-stimulated microglia (Fig. 5A–C). These observations substantiated that peimine promoted microglial M2 polarization *in vitro*.

Furthermore, Western blot results revealed that TLR4/NF-κB/HIF-1α signaling pathway was activated by LPS, with increased levels of TLR4, MyD88, nuclear NF-κB p65 and HIF-1α, and increased phosphorylation levels of IκB-α and NF-κB p65, which was abolished by peimine administration (Fig. 5D). In aggregate, our data validated that peimine treatment promoted microglial M2 polarization by inactivating the TLR4/NF-κB/HIF-1α signaling pathway.

3.6. Peimine suppressed the apoptosis of primary neurons induced by the conditioned medium of LPS-treated microglia

To investigate whether peimine affected the apoptosis of primary neurons induced by the conditioned medium of LPS-treated microglia, primary mouse hippocampal neurons were isolated based on the previously reported method and incubated with the conditioned medium from microglia [27]. CCK-8 data showed that the conditioned medium of LPS-treated microglia decreased neuronal viability, which was attenuated by peimine dose dependently (Fig. 6A). TUNEL staining was applied to detect neuronal apoptosis, and the results supported anti-apoptotic effects of peimine (Fig. 6B). Moreover, Bax expression was largely increased in the primary neurons treated with the conditioned medium of LPS-treated microglia, while and Bcl-2 was decreased (Fig. 6C). Alterations of these apoptosis-related proteins were weakened when the microglia were treated with peimine (Fig. 6C). These findings demonstrated that peimine suppressed the apoptosis of hippocampal neurons induced by LPS-treated microglia, with higher-dose of peimine having stronger effects.

4. Discussion

AED resistance is a major challenge affecting a significant proportion of epilepsy patients [28]. However, the etiology of DRE is complex with multifactorial influences [29]. Among them, the role of neuronal injury and neuroinflammation in the pathogenesis of DRE has received extensive attention. It has been reported that the KA model is one of the most commonly used to identify the underlying mechanisms of epilepsy genesis [30]. KA-induced DRE models simulate the key pathological features of human TLE, such as the occurrence of drug-resistant spontaneous seizures and hippocampal sclerosis [31]. After KA injection, three different time frames

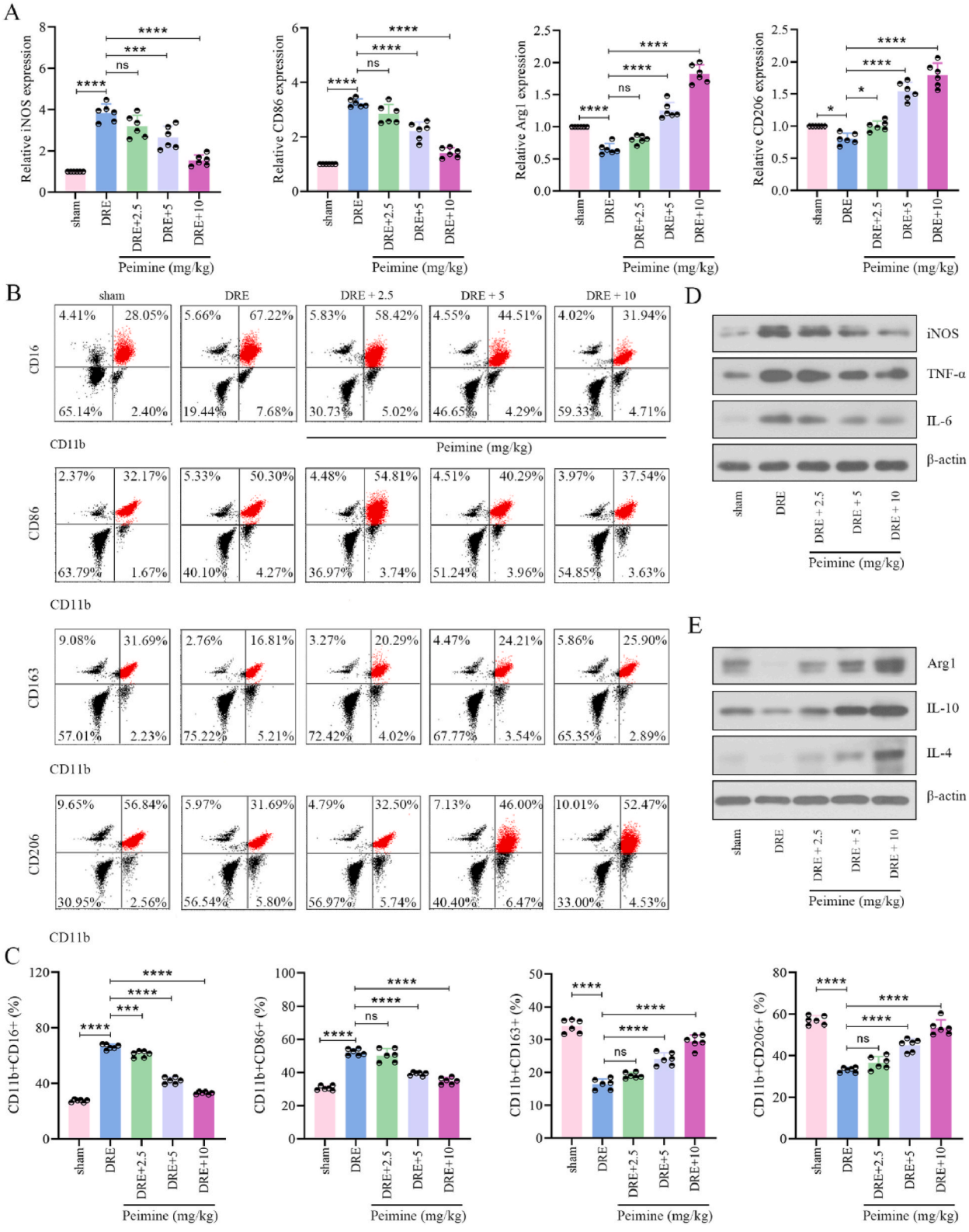

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Fig. 3. Peimine promoted an M1-to-M2 microglial phenotype shift in hippocampal microglia of DRE rats. (A) The expression of iNOS, CD86, Arg1 and CD206 in hippocampal tissues of rats was detected by qRT-PCR. (B–C) Flow cytometry was performed to detect the proportion of M1-phenotype microglia (CD11b + $CD16^+$ and CD11b + $CD86^+$) and M2-phenotype microglia (CD11b + CD163+ and CD11b + CD206+) in rat hippocampus. (D–E) The levels of M1 polarization markers (iNOS, TNF-α and IL-6) and M2 polarization markers (Arg1, IL-4 and IL-10) in rat hippocampus were detected by Western blot. *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$.

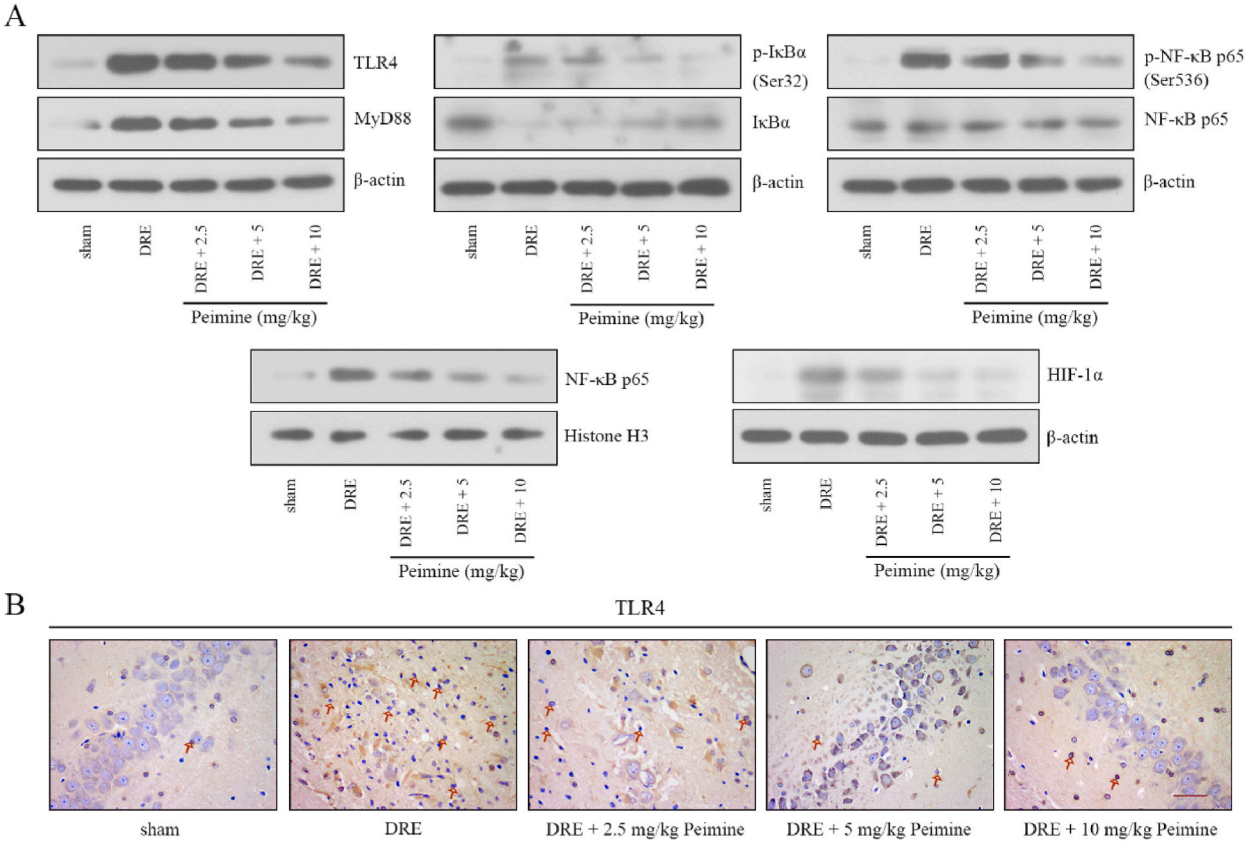

Fig. 4. Peimine inactivated the TLR4/NF-κB/HIF-1α signaling pathway in hippocampus of DRE rats. (A) Western blot was applied to detect the levels of TLR4, MyD88, IκBα, *p*-IκBα (Ser32), NF-κB p65, p-NF-κB p65 (Ser536), NF-κB p65 (nucleus) and HIF-1α in rat hippocampus. (B) The expression of TLR4 in CA3 region of rat hippocampus was measured by IHC (scale bar = 50 μm; the arrows represented TLR4-positive cells).

could be identified in the EEG recordings. The EEG is characterized by persistent seizure activity (status epilepticus, SE) lasting several hours, then a latent phase with sporadic low voltage spike or spike-and-wave activity, followed by a chronic epileptic period with SRS originating in the hippocampus [32]. In addition, growing studies have shown that microglial activation is associated with DRE. After a seizure, microglial cells remain morphologically activated for a long time [33,34]. Secretion of pro-inflammatory cytokines from microglia promotes neuroinflammation [35]. LPS is a well-known agonist that induces microglia activation [36]. Therefore, these models are suitable for studying the pathophysiology of DRE *in vivo* and *in vitro*.

In the present study, a KA-induced rat DRE model and LPS-activated BV-2 microglia were used to investigate the potential neuroprotective effects of peimine. Peimine is one of active components of Chinese traditional medicine Fritillaria [37]. Numerous studies introduced that peimine showed antioxidant, anti-inflammatory and pain suppressing effects in multiple diseases [8,12]. Therefore, we investigated the possible effects of peimine on DRE rats by administering different doses. In this study, we found that KA caused strongly SRS and epileptiform spike in rats, whereas, the epileptic behaviors of DRE rats were markedly attenuated by peimine in a dose-dependent manner. These results suggested that peimine may show therapeutic effects in the pathogenesis of DRE.

Hippocampal damage more frequently occurs in DRE patients and is mainly associated with severe neuronal loss [38,39]. Accordingly, we focused on the role of peimine in hippocampal injury. The DRE rats showed a decrease in NeuN-positive cells and an increase in FJB-positive cells and TUNEL-positive cells, suggesting that neuronal loss occurred. Whereas, peimine-treated DRE rats exhibited the opposite phenotype. In addition, augmented mossy fiber sprouting was observed in DRE rats, and peimine administration significantly alleviated it. Next, we investigated the effects of peimine on the apoptosis of primary hippocampal neurons induced by LPS-treated microglia. As illustrated, LPS-treated microglia decreased neuronal viability and induced apoptosis, which was reversed by peimine treatment. These findings confirmed that peimine showed neuroprotective effects during DRE.

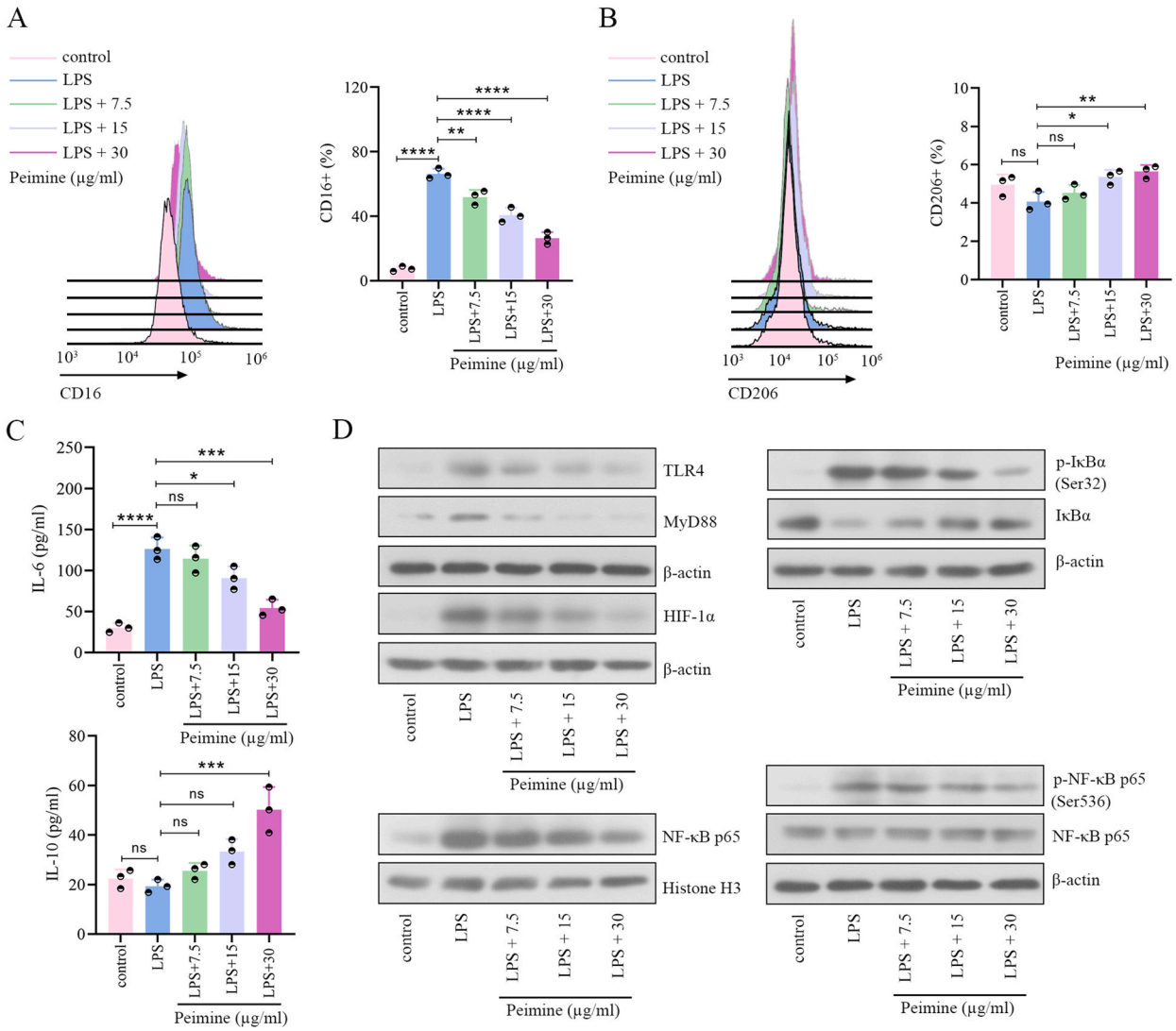

Fig. 5. Peimine promoted microglial M2 polarization by inhibiting the TLR4/NF-κB/HIF-1α signaling pathway in LPS-treated microglia. (A–B) Flow cytometry was performed to detect the proportion of M1-phenotype microglia ($CD16^{+}$) and M2-phenotype microglia (CD206+) in LPS-treated BV-2 cells. (C) The contents of IL-6 and IL-10 were determined by ELISA. (D) Western blot was employed to examine the levels of TLR4, MyD88, IκBα, *p*-IκBα (Ser32), NF-κB p65, p-NF-κB p65 (Ser536), NF-κB p65 (nucleus) and HIF-1α. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Neuroinflammation is an important factor in the pathophysiology of epilepsy [40]. Notably, peimine suppressed inflammation in mouse chondrocytes by inhibiting the MAPK pathway [10]. Moreover, peimine repressed pro-inflammatory cytokine secretion through inhibiting NF-κB and MAPK pathways in LPS-induced macrophages [6]. Importantly, microglia mediate multiple aspects of neuroinflammation, which is an important pathological process in central nervous system diseases, including epilepsy [13,41]. The microglial phenotype shift from M1 to M2 is considered an effective treatment strategy for DRE [16]. Of note, *in vivo* and *in vitro* results showed the M1 phenotype of microglia was activated and M2 phenotype of microglia was inhibited during DRE. However, peimine administration promoted microglial polarization to the M2 phenotype in a dose-dependent manner, which may contribute to its anti-inflammatory effects.

TLR4 can activate microglia via inducing NF-κB/MyD88/TRIF signaling pathway, resulting in inflammatory responses [18,42]. Inhibition of the TLR4/NF-κB pathway may trigger polarization of the microglia to the M2 phenotype [43]. We then investigated the underlying mechanism by which peimine promoted microglial polarization to the M2 phenotype. *In vivo* analysis suggested that DRE induced the activation of TLR4/NF-κB/HIF-1α pathway. Whereas, peimine administration significantly inactivating the TLR4/NF-κB/HIF-1α pathway in a dose-dependent manner, which was consistent with the *in vitro* data.

In summary, our findings demonstrate that peimine promotes microglia polarization toward M2 phenotype through suppressing the TLR4/NF-κB/HIF-1α signaling pathway, thus attenuating DRE (Fig. 7). These results highlight the therapeutic significance of peimine in the treatment of neurodegenerative diseases.

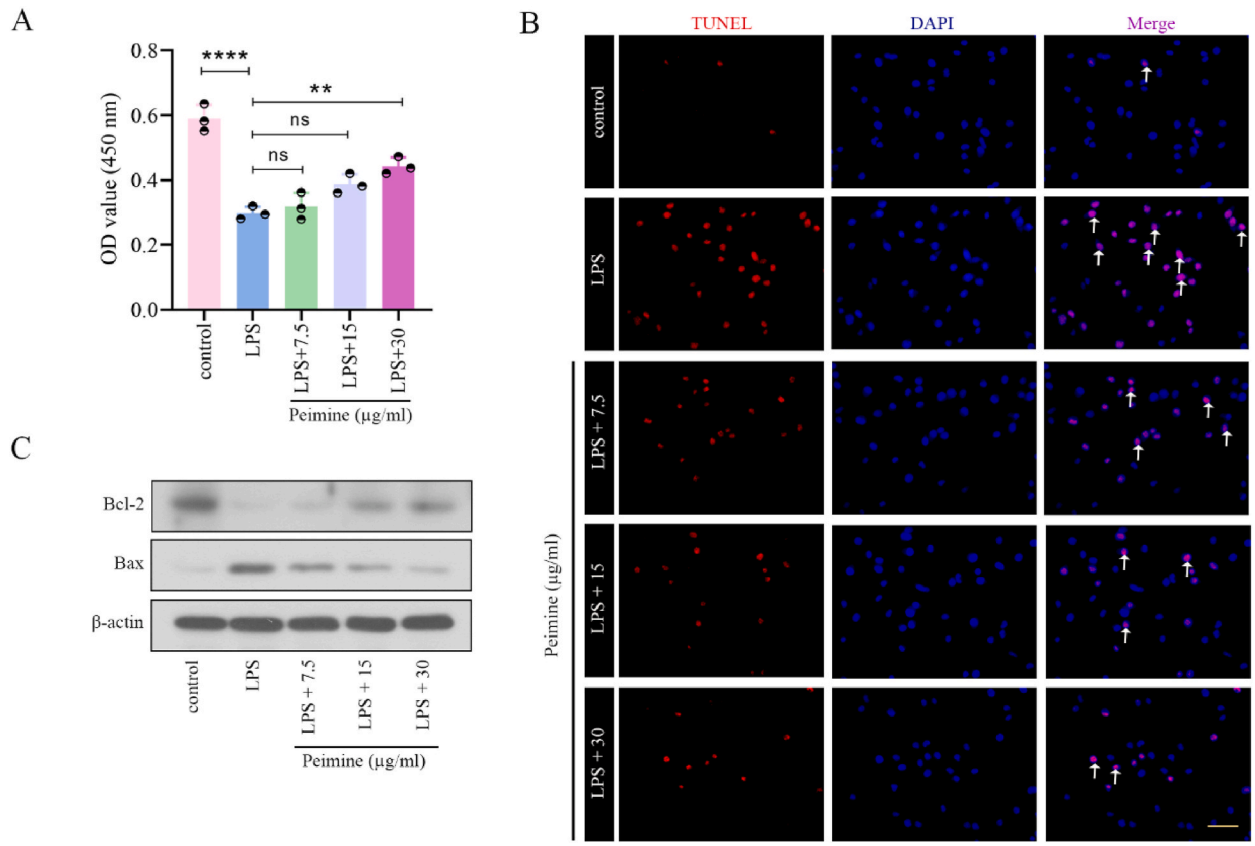

Fig. 6. Peimine suppressed the apoptosis of primary neurons induced by LPS-treated microglia. (A) CCK-8 assay was used to detect the viability of neurons. (B) TUNEL staining was applied to measure neuronal apoptosis (scale bar = 50 μm). (C) Western blot was used to measure the protein expression of Bax and Bcl-2 in neurons. **, $p < 0.01$; ****, $p < 0.0001$.

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Data availability statement

Data are available upon reasonable request.

CRediT authorship contribution statement

Chongchong Liu: Writing – original draft, Formal analysis, Data curation. **Jiangyan Sun:** Resources, Methodology, Investigation. **Xiaoming Shen:** Supervision, Conceptualization. **Shefang Li:** Investigation, Formal analysis. **Sha Luo:** Funding acquisition. **Na Chen:** Validation, Formal analysis. **Yunke Zhang:** Writing – review & editing, Supervision, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e34987.

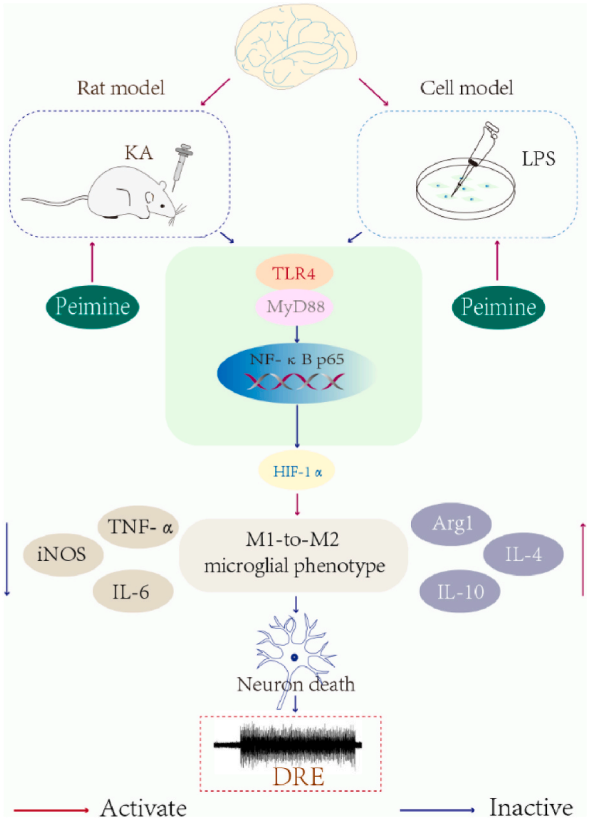

Fig. 7. Proposed scheme of peimine in DRE. Peimine promotes microglia polarization toward M2 phenotype, exhibiting a therapeutic role during DRE via inhibiting TLR4/NF-κB/HIF-1α pathway *in vivo* and *in vitro*.

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